STEAROYL-CoA DESATURASE GENE PROMOTER

FIELD OF THE INVENTION

The present invention relates generally to gene promoters and the use thereof.

BACKGROUND OF THE INVENTION

Stearoyl-CoA desaturase (Scd) is a lipid metabolic enzyme that catalyzes the insertion of a double bond between carbons 9 and 10 in the saturated fatty acids (SFA), palmitoyl-CoA and stearoyl-CoA, to generate the monounsaturated fatty acids (MUFA), palmitoleoyl-CoA and oleoyl-CoA, respectively. MUFA's play important roles in many processes including energy metabolism (triacylglycerol storage in adipose tissue) (Berry, 1997), membrane fluidity (Kates *et al.*, 1984; Tiku *et al.*, 1996), anti-oxidation (Parthasarathy *et al.*, 1990; Diplock *et al.*, 1988), signal transduction (Diaz-Guerra *et al.*, 1991; Gyorfy *et al.*, 1997; Kim *et al.*, 1999), apoptosis (de Vries *et al.*, 1997; Kasai *et al.*, 1998), and senescence (Fukuchi-Mizutani *et al.*, 1995; Kumar *et al.*, 1999; Lee *et al.*, 1999). Moreover, a critical role in differentiation is evidenced by the lack of development of the sebaceous and meibomian glands in the asebia mutant mouse, due to loss of *SCD1* function (Zheng *et al.*, 1999). Altered *SCD* gene expression is associated with various diseases such as cancer (Hrelia *et al.*, 1994; Li *et al.*, 1994), obesity (Jones *et al.*, 1996), and aging (Kumar *et al.*, 1999; Lee *et al.*, 1999). The pleiotropic nature of MUFA's requires complex regulation of *SCD* that includes 1) hormones such as T3 (Waters *et al.*, 1997a), insulin (Kasturi and Joshi, 1982; Prasad and Joshi, 1979; Waters and Ntambi, 1994) and estradiol (Lippiello *et al.*, 1979), 2) dietary factors such as fasting (Oshino and Sato, 1972), carbohydrate (Prasad and Joshi, 1979; Waters and Ntambi, 1994; Jones *et al.*, 1998), vitamin A (Miller *et al.*, 1997), polyunsaturated fatty acids (PUFA's) (Jeffcoat and James, 1978; Bossie and Martin, 1989; Sessler *et al.*, 1996; Ntambi *et al.*, 1996; Waters *et al.*, 1997b; Ntambi, 1999), the metals iron (Rao *et al.*, 1983) and copper (Wahle and Davies, 1975; Sreekrishna and Joshi,

1980), and peroxisomal proliferators, (Miller and Ntambi, 1996; Kurebayashi *et al.*, 1997), and 3) environmental factors such as temperature (Kasai *et al.*, 1976; Tiku *et al.*, 1996; Trueman *et al.*, 2000). Furthermore, the rat Scd protein is shortlived (t1/2=3-4 hr) due to the action of an integral membrane-bound protease in the endoplasmic reticulum (Heinemann and Ozols, 1998).

In order to gain insight into the complex regulation of *SCD* genes, nucleotide sequences have been isolated from many organisms including plant, bacteria, protozoa, yeast, tick, fish, hamster, sheep, cow, rat, mouse, and human (Sakamoto *et al.*, 1994; Ideta *et al.*, 1995; Ward *et al.*, 1997; Fukuchi-Mizutani *et al.*, 1998; Tocher *et al.*, 1998; Zheng *et al.*, 1999). At the level of amino acid and nucleotide sequence, the *SCD* gene family exhibits both similarities and differences across the phylogenetic kingdom (Shanklin *et al.*, 1994). The similarities are 1) the high levels of identity and similarity of the open reading frames (ORF's) among the various organisms, 2) the presence of conserved amino acids (and their spacing) involved in catalysis of the Δ9 desaturation reaction, and 3) the presence of a long 3' untranslated region (UTR) in the vertebrate genes. The major difference is the variable number of *SCD* genes between different species. Those organisms with a single structural gene include cyanobacteria (Sakamoto *et al.*, 1994), the yeast s. cerevisiae (Stukey *et al.*, 1989), sheep (Ward *et al.*, 1997), and human (Zhang *et al.*, 1999) whereas those with more that one structural gene include mouse (Ntambi *et al.*, 1988; Kaestner *et al.*, 1989) and rat (Mihara, 1990), suggesting that *SCD* has undergone duplication and expansion within the rodent lineage. The reason for the multiple *SCD* genes in rodent is unclear, although there is recent evidence to suggest that mouse *SCD*1 and *SCD*2 have different substrate specificities (Kim and Ntambi, 1999). This hypothesis is supported by the finding of tissue specific expression of the rodent *SCD* isoforms (Ntambi *et al.*, 1988; Kaestner *et al.*, 1989; Mihara, 1990; Zheng *et al.*, 1999), whereas the human (Zhang *et al.*, 1999) and sheep (Ward *et al.*, 1997) *SCD* are ubiquitously expressed. Further species-specific regulatory mechanisms are indicated by the use tandem polyadenylation signals by human *SCD* to generate 2 alternate mRNA

transcripts (Zhang *et al.*, 1999), each encoding the identical enzyme, whereas the rodent *SCD* isoforms generate a single mRNA transcript (Ntambi *et al.*, 1988; Kaestner *et al.*, 1989; Mihara, 1990).

Due to the diverse processes in which *SCD* functions, the regulation of *SCD* gene expression is involves both transcriptional and post-transcriptional mechanisms (Ntambi, 1995; Sessler *et al.*, 1996; Sessler and Ntambi, 1998; Kim and Ntambi, 1999; Tabor *et al.*, 1999). Studies of transcriptional regulation have focussed on functional analysis of promoters, these having been isolated from yeast s. cerevisciae *OLE1*(Stukey *et al.*, 1990) and h. capsulatum *OLE1*(Tosco *et al.*, 1997), mouse *SCD*1 (Ntambi *et al.*, 1988) and *SCD*2 (Kaestner *et al.*, 1989), rat *SCD*1 (Mihara, 1990), and sheep *SCD* (Ward *et al.*, 1997). The mouse *SCD*1 promoter has been extensively analyzed and cis-elements identified that regulate transcription (Ntambi, 1995; Kim and Ntambi, 1999; Tabor *et al.*, 1999).

Activation of mouse *SCD*1 transcription during 3T3-L1 adipocyte differentiation requires binding sites for 1) the differentiation-induced transcription factor SREBP (Tabor *et al.*, 1999), and 2) the constitutive transcription factors NF-1 (Singh and Ntambi, 1998)and NF-Y (Tabor *et al.*, 1999). The promoter of *SCD*2 contains the same cis elements and also requires the SREBP and NF-Y binding sites (Tabor *et al.*, 1998; Tabor *et al.*, 1999). Peroxisome proliferators have differential effects on *SCD* mRNA accumulation. The PPAR-γ ligand clofibrate activates transcription of mouse *SCD*1 via a PPRE located in the promoter (Miller and Ntambi, 1996), an effect suggested to account for maintenance of membrane fluidity during peroxisome proliferator induced expansion of cellular membranes. In contrast, the PPAR-γ ligand troglitazone suppresses accumulation of mouse *SCD*1 mRNA, an effect which may account for its use as an anti-obesity agent (Kurebayashi *et al.*, 1997; Kim and Ntambi, 1999).

Due to the important role of unsaturated fatty acids (UFA's) in controlling fluidity of membranes and lipid stores, *SCD* gene expression is negatively regulated in vivo and in vitro by UFA's, both MUFA's (McDonough *et al.*, 1992; Meesters *et al.*, 1997) and PUFA's (Jeffcoat and James, 1978;

Ntambi, 1995; Sessler and Ntambi, 1998; Ntambi, 1999). UFA-mediated repression of *SCD* mRNA has tissue-specific mechanisms, being transcriptional in liver (Sessler and Ntambi, 1998; Ntambi, 1999), and post-transcriptional in adipose tissue (Sessler *et al.*, 1996). A functional PUFA response element has been identified in the mouse *SCD*1 (Waters *et al.*, 1997b; Tabor *et al.*, 1999) and *SCD*2 (Tabor *et al.*, 1999) promoters that mediates repression in cultured hepatocytes (Waters *et al.*, 1997b; Tabor *et al.*, 1999) treated with a variety of PUFA's. This region encompasses the essential SREBP and NF-Y binding sites, suggesting that the PUFA-mediated repression involves destabilization of the cooperativity between SREBP and NF-Y (Tabor *et al.*, 1999). Indeed, PUFA destabilize SREBP mRNA (Xu *et al.*, 1999; Yahagi *et al.*, 1999) suggesting that part of PUFA mediated supression of *SCD* mRNA is due to loss of the critical activator SREBP. In adipose tissue, on the other hand, PUFA induces the destabiliztion of *SCD* mRNA , possibly mediated by AUUUA destabilization sequences in the long 3'UTR (Sessler *et al.*, 1996), a common feature of all vertebrate *SCD* genes, including human.

In order to understand the role of *SCD* in human health and disease, we recently isolated the full length human *SCD* cDNA and structural gene, demonstrating the high homology to the mouse *SCD* genes, particularly with respect the ORF, intron-exon junctions, and the long 3' UTR (Zhang *et al.*, 1999). Unlike mouse and rat which have at least 3 *SCD* loci, human has only 2 loci, one being the structural gene and the other a fully processed pseudogene (Zhang *et al.*, 1999). In this report, we characterize the human *SCD* promoter from the structural gene on chromosome 10. The human *SCD* promoter is similar to the mouse *SCD* promoters in that the PUFA response region is conserved and contains within it the critical SREBP and NF-Y binding sites required for mouse *SCD*1/*SCD*2 transcription. Further, we demonstrate that the CCAAT box (putative NF-Y binding site) binds nuclear proteins and is required for transcriptional activation in cultured keratinocytes. A minimal promoter, bearing this region, is responsive to UFA mediated transcriptional repression, as well as transcriptional activation by SFA.

Finally, many putative transcription factor binding sites are identified that may play a role in regulating the diverse functions of *SCD*.

SUMMARY OF THE INVENTION

The object of the present invention is to provide a promoter that causes high gene expression. Another object is to provide a promoter that can be used for selective transgenic expression into various tissues, such as the skin. The present invention provides a promoter of the stearyl-CoA desaturase gene, functional moieties of this gene, fragments, and variants thereof. Various embodiments provide, nucleic acid constructs and vectors that contain such sequences, their functional moieties, fragments, and variants, and the uses thereof.

In yet another embodiment the invention pertains to a pharmaceutical composition for treating a disease, said composition comprising a promoter of the stearoyl-CoA desaturase, a functional moiety, or a variant sequence thereof.

Other features and advantages of the present invention will be apparent from the detailed description of the invention and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: Characterization of the 5' end of the human *SCD* gene.

Sequence of the 5' end of the human *SCD* gene. Numbering is relative to the translation initiation codon. Amino acids are indicated with single letter code. Start site of transcription in hair follicle tissue is boxed. Minor transcription sites common to hair follicles and HaCaT cells is dashed boxed. The FSE2 is double boxed. Termination of *SCD* cDNA clones 34 (filled arrowhead) and 45 (open arrowhead) are indicated. Arrow indicates the most 5' extent of RNase-protected fragment in HaCaT keratinocyte cells. The core sequence of transcription factor binding sites, as determined by the Matinspector Professional search tool using the Transfac database, are

underlined, with arrowheads indicating orientation. Restriction enzyme sites are indicated by caret marks above the sequence. Bold letters indicate the 5' extent of the clones used for reporter assays.

FIGURE 2 Comparison of the proximal promoter of mouse *SCD1* and human *SCD*

Sequence alignment of the 5' UTR and proximal promoter of mouse *SCD1* (Ntambi *et al.*, 1988) and human *SCD*. Numbering is relative to translation initiation site. Colon indicates identity between sequences. Transcription factor binding sites unique to human, as well as human sequence, are in italics. Transcription factor binding sites unique to mouse, as well as mouse sequence, are in regular font. Transcription factor binding sites common to mouse and human are bold. Arrowheads indicate orientation of transcription factor binding sites. Dashed box indicates FSE2. Solid boxes indicate transcription start site of mouse *SCD1* in 3T3-L1 cells (Ntambi *et al.*, 1988) and transcription start site of human *SCD* in human hair follicles.

FIGURE 3: Determination of transcription initiation site

15 μg of total RNA from human hair follicles (lane 1) or HaCaT keratinocytes (lane 2) was hybridized with a 400 nt *SCD* riboprobe (302 nt is *SCD* sequence) at 42°C. After digestion of single-stranded RNA with RNase A/T1, the protected fragments were resolved along with a sequencing ladder of the probe-containing vector NPA2-pCR2.1. Filled arrowheads indicate major sites of transcription. Open arrowhead minor transcription start site common to hair follicles and HaCaT keratinocytes. Sizes (nt) of the protected fragments are indicated.

FIGURE 4: Function analysis of human *SCD* Promoter

Upper Left: a panel of deletion constructs. Restriction sites are indicated. Sequence numbering is relative to translational start site. c1- is the reverse orientation of c1. Upper Right: promoter activity of constructs transfected into HaCaT cell line. Data is representative of at least two independent experiments. Constructs were transfected in duplicate, cell lysates harvested 48 h post-transfection, and readings performed in triplicate. Values are plotted as a ratio of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase.

FIGURE 5: Fine Deletion Analysis of the human *SCD* promoter

Left: panel of deletion constructs. Restriction sites and 5' ends of primer sites are indicated. Right: promoter activity of constructs transfected into HaCaT cell line. Data is representative of at least two independent experiments. Constructs were transfected in duplicate, cell lysates harvasted 48 h post-transfection, and readings performed in triplicate. Values are plotted as a ratio of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase. Lower panel: promoter region showing location of c9, c10, c6 (bold), location and orientation of transcription factor binding sites (underlined), nucleotides (boxed) subjected to site directed mutagenesis in Figure 6, and oligonucleotides (lowercase) used for EMSA in Figure 7.

FIGURE 6: Site Directed Mutagenesis of SP-1 site and CCAAT box

Using c9 as a template, site directed mutagenesis of the SP-1 site (-551) and the CCAAT box (-501) were carried out as indicated in Example 6. Promoter activity of constructs transfected into HaCaT cell line is shown. Data is representative of at least two independent experiments. Constructs were transfected in duplicate, cell lysates harvasted 48 h post-transfection, and readings performed in triplicate. Values are plotted as a ratio of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase. "Control" is transfection without plasmid DNA.

Figure 7: EMSA of CCAAT box binding proteins

Nuclear proteins from HaCaT keratinoccytes were mixed with ^{32}P-labeled CCAAT oligonucleotide, with and without competitor oligonucleotide, and analyzed using non-denaturing gel electrophoresis as described in Example 4. Sense strand of the double stranded oligonucleotides used for EMSA are indicated in red in Figure 6; AP-2, -695 to –575; SP-1, -559 to –539; CCAAT, -509 to –489. Arrowhead indicates the band demonstrating specific CCAAT box binding proteins. The bar indicates bands that bind putative CCAAT box binding proteins with partial specificity.

Figure 8: Comparison of the PUFA-response region of mouse and human SCD Genes

Sequence alignment of the PUFA-response region mouse *SCD1* (Ntambi *et al.*, 1988), mouse *SCD2* (Kaestner *et al.*, 1989), and human *SCD*. Numbering is relative to translation initiation site for all three genes. Colon indicates identity between sequences. All transcription factor binding sites and orientations (arrowheads) indicated are conserved among all three genes. SRE element is indicated in bold.

FIGURE 9: *SCD* promoter activity in response to fatty acid treatment

24 hours following transfection of c9 into HaCaT keratinocytes, the cells were exposed to 100 uM of fatty acid/BSA for 24 hours. Cells were harvested and analyzed for luciferase activity. Data is representative of at least two independent experiments. Constructs were transfected in duplicate, cell lysates harvasted 48 h post-transfection, and readings performed in triplicate. Values are plotted as a ratio of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase.

DETAILED DESCRIPTION OF THE INVENTION

It is believed that one skilled in the art can, based upon the description herein, utilize the present invention to its fullest extent. The following specific

embodiments are to be construed as merely illustrative, and not limitive of the remainder of the disclosure in any way whatsoever.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Also, all publications, patent applications, patents, and other references mentioned herein are incorporated by reference.

The present invention relates to a DNA whose nucleotide sequence comprising a sequence selected from the sequence from about 145 to about 4152 in the sequence listing in Figure 1 which possesses promoter activity. The phrases "sequence selected from the base sequence from about 145 to about 4152 in the sequence listing in Figure 1" and "sequence selected from the base sequence from about 193 to about 4152 in the sequence listing in Figure 1" includes not only the entire sequences, but portions of these sequence that have promoter activity. This definition also includes functional moieties of the promoter. Thus, the present invention is not restricted to sequences selected from the base sequence from about 145 to about 4152 in the sequence listing in Figure 1 (i.e. the entire sequence, and functional moieties thereof) but also comprises variants which possess promoter activity. Variants of this nature comprise, for example, deletions, additions, insertions and/or substitutions of one or more bases, preferably of from approximately 1 to approximately 50, in particular of from approximately 1 to approximately 25, especially of from approximately 1 to approximately 5, bases from a sequence selected from the base sequence from about 145 to about 4152 in the sequence listing in Figure 1. Thus, a "variant" as termed here, is a modified sequence from the base sequence from about 145 to about 4152 in the sequence listing in Figure 1 that still has promoter activity. Modifications can be made by various techniques that are known to the skilled artisan. An example of such a technique is given in Sambrook, J. et al., MOLECULAR CLONING. LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory Press (1989).

The promoter activity can be readily measured, for example, using the luciferase assay described herein and/or from the CAT or b-gal reporter assay methods described in Sambrook, J. et al., MOLECULAR CLONING. LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory Press (1989).

The present invention furthermore also relates to a nucleic acid construct which comprises a) at least one nucleic acid sequence of the promoter according to the invention (component a)) and, where appropriate, b) at least one effector gene (component b)), with the transcription of this effector gene being activated by component a). Component a) is preferably located upstream of component b).

A nucleic acid construct according to the invention is preferably composed of DNA. The term "nucleic acid construct" is understood as meaning artificial structures which are composed of nucleic acid and which can be transcribed in the target cells. They are preferably inserted into a vector, for example into non-viral vectors, such as plasmids, or viral vectors. The skilled artisan is familiar with the preparation of non-viral vectors and of viral vectors. Such preparation is explained, for example, in Sambrook, J. et al., MOLECULAR CLONING. LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory Press (1989).

The present invention also relates to host cells which harbor a nucleic acid construct according to the invention. Such host cells can be prokaryotic or eukaryotic, and advantageously are skin cells such as fibroblasts and keratinocytes.

The present invention also relates method for determining whether an agent effects (e.g., increases or decreases) the expression level of the effector gene (e.g., stearoyl-CoA desaturase such as human stearoyl-CoA desaturase) in a host cell of the present invention, which comprises the steps of (i) contacting the agent under suitable conditions with such a host cell expressing such effector gene at a known level; and (ii) determining whether the effector gene expression level increases or decreases after cellular contact with the agent, thereby determining whether the agent increases or

decreases the expression level of the effector gene in skin cells already expressing the same. In one embodiment, the effector gene is a luciferase, β-galatosidase, or a cloramphenicol acetyltransferase gene.

The invention furthermore relates to a nucleic acid construct in which the promoter sequence according to the invention is combined with another target cell-specific, virus-specific, metabolism specific or cell cycle-specific promoter sequence and with at least one effector gene, in which this combination of promoter sequences controls the activation of at least one effector gene.

In general, the choice of an effector gene, in accordance with the invention, depends on the disease to be treated with the gene construct. Examples of effector genes are those used for the therapy of skin diseases (such as acne, psoriasis, and rosacea), tumor diseases, leukemias, autoimmune diseases, allergies, arthritis, inflammations, organ rejections, graft versus host reactions, diseases of the blood coagulation system, cardiovascular diseases, anemia, infections or damage to the CNS and are described in detail in PCT Patent Applications WO 96/06940, WO 96/06938, WO 96/06941 and WO 96/06939.

A nucleic acid construct according to the invention can furthermore comprise two or more identical or different effector genes which are linked to each other by way of promoter sequences or internal ribosomal entry sites (IRES). Examples of these are given in the above mentioned patent applications. Methods for preparing these constructs are well known to the skilled artisan, as for example, described in the above mentioned patent applications which are incorporated by reference in their entireties.

A nucleic acid construct according to the invention can be used, for example, to express a gene 1) specifically in a skin cell, 2) specifically in a skin cell and also in a metabolism specific manner, 3) specifically in a skin cell and also cell cycle- specifically and 4) specifically in a skin cell and virus-specifically (and optionally cell cycle or metabolic specific). The controlled gene preferably encodes a pharmacologically active compound or an enzyme which cleaves an inactive precursor of a drug, thereby forming an active drug.

Preference is given to using a nucleic acid construct according to the invention to prepare a pharmaceutical composition (drug), for treating at least one of the above mentioned diseases, with the preparation of a pharmaceutical generally comprising the cloning of the nucleic acid construct into a suitable vector, which is then, for example, administered to the patient. A skilled artisan is familiar with other ways of using a promoter and these other ways are possible according to the invention. A skilled artisan also will appreciate creation and modification of various nucleic acid constructs according to the invention.

The pharmaceutical compositions contemplated are intended for parenteral, topical, oral or local administration and generally comprise a pharmaceutically acceptable carrier and an amount of the active ingredient sufficient to reverse or prevent the bad effects of a disease state. The carrier may be any of those conventionally used and is limited only by chemico-physical considerations, such as solubility and lack of reactivity with the compound, and by the route of administration.

Examples of pharmaceutically acceptable acid addition salts for use in the present inventive pharmaceutical composition include those derived from mineral acids, such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric and sulfuric acids, and organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, p-toluenesulphonic acids, and arylsulphonic, for example.

The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, carriers or diluents, are well-known to those who are skilled in the art and are readily available to the public. It is preferred that the pharmaceutically acceptable carrier be one that is chemically inert to the active compounds and one that has no detrimental side effects or toxicity under the conditions of use.

The following examples are intended to describe the invention in more detail without limiting it.

EXAMPLE 1: Genomic Fragment Isolation

Isolation of the λ phage encompassing the 5' end of the *SCD* gene (4 kb of promoter, exon 1, intron 1, exon 2, and part of intron 2) from a chromosome 10-specific genomic library was previously obtained using standard techniques (Zhang *et al.*, 1999). The 5.6 kb insert was cloned as an EcoRI fragment into pBlueScript Stratagene, La Jolla, CA, USA) (clone 19) and subsequently used to generate both nuclease protection assay probes and luciferase reporter assay constructs as described below.

EXAMPLE 2: Luciferase Reporter Construction

To generate p(-3348/-150)*SCD*-luc (c1), clone 19 was digested with *Pst*I and *Sac*II and blunted with T4 polymerase. All sequences herein are numbered relative to the translation start site, designated +1. The 3.2 kb fragment containing only the 5' end of the gene was cloned into a blunted *Hin*dIII site of the luciferase vector pGL3-basic (Promega, Madison, WI, USA). p(-2328/-150)*SCD*-luc (c2) and p(-1295/-150)*SCD*-luc (c4) were generated by digesting the clone 19 with *Taq*I/*Sac*II and *Kpn*I/*Sac*Ii, respectively, and the resulting fragments were subcloned into a blunted *Hin*dIII site of pGL3-basic. p(-1537/-150)*SCD*-luc (c3) and p(-882/-150)*SCD*-luc (c5) were subsequently generated by digesting c1 with *Sac*I and *Sma*I, respectively. The resulting fragments were religated using T4 ligase. p(-461/-150)*SCD*-luc (c6) was generated by digesting c4 with *Eae*I/*Nco*I and the resulting fragment was ligated to blunted *Nco*I/*Hin*dIII sites of pGL3-basic. p(-270/-150)*SCD*-luc (c7) was created by digesting c4 with *Hin*cII and *Nco*I. The resulting fragment was subcloned into *Nco*I/*Hin*dIII digested, blunted, pGL-3 basic. p(-753/-150)*SCD*-luc (c8), p(-609/-150)*SCD*-luc (c9) and p(-496/-150)*SCD*-luc (c10) constructs were assembled by polymerase chain reaction using one of the three specific sense oligonucleotides (bp -753 to -732, c8), 5'-GGTTCACCACTGTTTCCTGAGA-3'; (-609 to -688, c9) 5'-GATGCCGGGCAGAGGCCCAGCG -3';(–496 to -474, c10), 5'-GGCAACGGCAGGACGAGGTGGCA-3'; and a common antisense oligonucleotide (-166 to -145) 5'-CCGCGGTGCGTGGAGGTCCCCG-3'. All PCR reactions were conducted with proofreading Turbo *PFU* DNA

polymerase (Stratagene, La Jolla, CA, USA), and the original c5 construct was used as template. Amplification products were phosphorylated with T4 kinase and subcloned into the *Hind*III blunted site of pGL-3 basic. The sequence of the PCR products were examined for accuracy using the dideoxynucleotide chain termination method.

EXAMPLE 3: RNase Protection Analysis

Total RNA was extracted from human hair follicle biopsies and cultured HaCaT keratinocytes (Boukamp *et al.*, 1988) using RNAzol (TEL-TEST Inc., Friendswood, TX, USA). A *SCD* fragment was amplified with PCR, using clone 19 as a template with the following primers: sense (-275 to -256), 5'GCCAGTCAACTCCTCGCACT3'; antisense (+7 to +27), 5'ATCGTCCTGCAGCAAGTGGGC3'. This resulted in a 302 bp fragment (nucleotide -275 to +27) which was subcloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA, USA). The plasmid (NPA1-pCR2.1) was further digested by Xba1 and EcoRV to release unnecessary vector sequence between the insert and the T7 promoter and religated with T4 ligase. The final construct (NPA2-pCR2.1) was linearized by H*ind*III and a labeled antisense RNA (400 bp) was synthesized from the T7 promoter of the plasmid using T7 RNA polymerase (Ambion, Austin, TX, USA). Following synthesis, the full-length probe was gel purified. Total RNA (15ug per sample) from human hair follicles and HaCaT keratinocytes was hybridized to the labeled transcript for 16h at 42°C. The RNA samples were digested with an RNase mixture (Ambion, Austin , TX, USA), and the sizes of the protected RNA were determined by electrophoresis on a 5% denaturing polyacrylamide gel, using a DNA sequencing ladder generated from NPA2-pCR2.1.

EXAMPLE 4: Nuclear Extracts and Electrophretic Mobility Shift Assays

Nuclear extracts of HaCaT keratinocytes were prepared using standard procedures (Dignam *et al.*, 1983). Protein concentrations of the extract were determined with BCA assay (Pierce, Rockford, IL, USA). Electrophoretic mobility shift assays were performed with a Gel Shift assay system according

to the manufacture's instruction (Promega, Madison, WI, USA). Briefly, hybridization was performed at room temperature for 20 min in a volume of 20 ul consisting of 0.1 pmol of probe (70, 000 cpm) and 5 ug of nuclear extract. The gel shift mixture was incubated for 20 min at room temperature, and samples were immediately electrophoresed at 150V for 3 h on a 5% nondenaturing acrylamide gel using 0.5 x TBE running buffer (34 mM Tris.borate, 0.75 mM EDTA) prerun at 100 V for 100 min. The gel was then dried for autoradiography. For competition studies, radioinert DNA competitor was added as a 100-fold molar excess and preincubated with the nuclear extract at room temperature for 5 –10 min. The ^{32}P-labeled DNA was then added to the mixture and incubated at room temperature. The oligonucleotides used for EMSAs were as follows: AP-2 (sense, -595 to –575), 5'-GCCCAGCGGCGGGTGGAAGAG-3'; AP-2 (antisense), 5'-CTCTTCCACCCGCCGCTGGGC-3'; SP-1 (sense, -559 to -539), 5'-AACAGAGGGGAGGGGGAGCGA-3'; SP-1(antisense), 5'-TCGCTCCCCCTCCCCTCTGTT-3'; CCAAT (sense, -509 to -484), 5'GCGCCGAGCCAATGGCAACGG-3'; CAAT (antisense), 5'-CCGTTGCCATTGGCTCGGCGC-3'.

EXAMPLE 5: Transfection and Dual Luciferase Assay

The human keratinocyte cell line, HaCaT, was used as the recipient cells for transient transfection assays. The day prior to transfection, cells were seeded into 6 well plates at 400,000 cells per well. Transient transfections were performed with 2 ug of test plasmids using SuperFect Reagent (Qiagen, Valencia, CA, USA) according to the manufacture's protocol. As a control for transfection efficiency and cell density, HaCaT cells were co-transfected with a 50 ng of *Renilla* luciferase control reporter vector driven by the thymidine kinase promoter (pRL-TK). 48 h post-transfection, adherent cells were washed once with 3 ml of phosphate-buffered saline, dissolved in 500ul of cell culture lysis reagent (Promega, Madison, WI, USA), and harvested after 30 mins at room temperature. Luciferase assays were performed immediately using a Dual-Luciferase Reporter Assay System kit

(Promega, Madison, WI, USA) and measurements were performed using TD-20/20 luminometer (Promega, Madison, WI, USA) . The promoter activity was reflected by the ratio of firefly/*Renilla* luciferase for each construct. Triplicate readings were taken for each sample, all assays were performed in duplicate, and each experiment was repeated a minimum of two times.

EXAMPLE 6: Mutagenesis

Mutation in the binding sites for transcription factors SP-1 and CCAAT were introduced into the plasmid *c9*, containing the 0.5 kb *SCD* promoter subcloned into the blunted HindIII site of pGL-3 basic, using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Two double-stranded oligonuclotide primers containing the desired mutations are as follows (underlined base pairs denote mutant substitutions): 5'-AAGGAGAAACAGAGGAAAGGGGGAGCGAGGAGCTG-3' (SP-1), and 5'-AGCAGATTGCGCCGAGAAAATGGCAACGGCAGGAC-3'(CCAAT).

EXAMPLE 7: Cell Culture

All reagents from Gibco BRL (Rockville, MD, USA) unless otherwise stated. HaCaT keratinocytes were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA), 2 mM L-glutamine, and 50 unit/ml penicillin/streptomycin. The cells were incubated in humidified incubators at 37°C and 5% CO_2. For fatty acid treatments, HaCaT cells were exposed to fatty acid/BSA conjugate (100 uM) for 24 hours prior to harvest. Stock solutions (2.75 mM) of fatty acid/BSA solutions were prepared as follows: 8.5 mg stearate (Sigma: S3381) was dissolved in 1 ml 0.1 M NaOH then heated to 90°C for dissolution. This solution was added dropwise to 6 ml of 10% fatty acid free BSA (Sigma: A7511) with stirring at 37°C. This solution was brought to 10 ml with dH2O, filtered through 0.22 uM filter, and stored at 4°C. The same procedure was followed for oleate (O3880), however heating was kept to a minimum. BSA solution used for controls was prepared following above procedure but without added fatty acids. Linoleate/BSA conjugate was purchased from

Sigma (L8384) and reconstituted to 3mM with dH2O. All stock solutions were diluted to 100 uM in cell culture media for application to cells.

EXAMPLE 8: DNA Sequencing

The DNA sequence in both directions was determined by Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT, USA) using Perkin Elmer Biosystems (Foster City, CA, USA) model 337 gel DNA sequencer and model 3700 capillary DNA sequencer.

EXAMPLE 9: Characterization of the 5'-region of the *SCD* gene

Previously we have shown that there are two *SCD* loci in human genome with one being the functional gene present on chromosome 10, and the other a pseudogene located on chromosome 17 (Zhang *et al.*, 1999). To obtain the upstream sequence of the functional *SCD* gene, chromosome 10-specific libraries were screened with a 1.9 kb fragment (from clone 34) containing the 5' end of the *SCD* cDNA (Zhang *et al.*, 1999). One of the genomic clones obtained from this screen had a 5.6 kb insert (Zhang *et al.*, 1999). Analysis of the insert indicated that there were approximately 4.0 kb upstream of the ATG translation start site.

Transcription factor binding sites were identified using the Matinspector Professional algorithm with the Transfac database v4.1 (Quandt *et al.*, 1995) using core similarity setting of 0.75 and matrix similarity setting of 0.85. Many classes of transcription factor binding sites were identified, consistent with the pleiotropic function of *SCD* genes (Fig. 1). Two TATA boxes are present, being located at –179 and –234. Binding sites for constitutive transcription factors involved in basal promoter activity included Stimulating Protein 1 (SP-1) (-1038,-930,-551,-314,-304), CCAAT box binding proteins Nuclear Factor-1 (NF-1) (-2743, -1872, -1744, -1651, -1241, -827, -502, -459, -244) and Nuclear Factor-Y (NF-Y) (-3981, -3898, -501, -458), X box-binding protein Regulatory Factor X-1 (RFX-1) (-733, -522, -495, -196), Octamer binding proteins Oct-1 (-1160, -427) and Oct-6 (-177), AP-2 (-2440, -1987, -802, -

695, -594, -194), Ets (-972, -212), cAMP Response Element Binding-Protein (CREB) (-1377, -1145, -454), and GC box-binding protein (-410). Although *SCD* is expressed in many tissues, its cellular pattern of expression within a tissue can be very specific, as seen in the mouse pilosebaceous unit (Zheng *et al.*, 1999). Consistent with this is the presence of tissue-specific transcription factor binding sites such as skin-enriched Winged-helix nude (Whn) (-2290, -942, -858, -811, -615, -291, -155), liver-enriched Hepatocyte Nuclear Factor (HNF)-3β (-2730, -2424), HNF-3/Forkhead (Fkh) Homolog 1 (-4048), HNF-3/Fkh Homolog 2 (-1958), HNF-4 (-256), and eye-enriched Pax-6 (-2016, -1858). Desaturases are highly regulated in tissues engaged in lipid metabolism such as liver, skin, and adipose tissue. Accordingly, binding sites for transcription factors that regulate lipid metabolic genes are present in the *SCD* promoter, these being Sterol Regulatory Element Binding-Protein (SREBP) (-4027, -3692, -3484, -3612, -2236, -2010), Peroxisome Proliferator-Activated Receptor (PPAR)-γ (-1186, -257), and CCAAT Enhancer Binding-Protein (C/EBP)-β (-2913, -1946, -1124, -441). Moreover, a sequence similar to the Fat Specific Element 2 (FSE2), common to many lipid metabolic genes, was found at –224 nt. Consistent with the alteration of *SCD* mRNA levels by steroid hormones is the presence of binding sites for members of the steroid hormone nuclear receptor superfamily including Vitamin D Receptor (VDR) (-324, -224; see Fig. X), Retinoic Acid Receptor (RAR)-α1 (-3080, -2228, -1904, -1762), Estrogen Receptor (ER) (-1738, -1359), Thyroid Hormone Receptor (T3R) (-2228), and Glucocorticoid Receptor (GR) (-1570). Consistent with a role in inflammation is the presence of Rel (-1689, -220), NF-kB (-2566, -2525, -1047, -1041, -868, -862, -727) , Interferon Regulatory Factor (IRF)-1 (-3612, -2521), IRF-2 (-3890, -2320), Interferon Stimulated Response Element (ISRE) (-561), and Signal Transducers and Activators of Transcription (STAT) (-1138) binding sites. That *SCD* may play a role in viral infection is suggested by the presence of binding sites for papilloma virus E2 protein (-214, -206) and BRLF1 protein of Epstein-Barr Virus (EBV) (-379). Finally, a role for *SCD* in proliferation and differentiation is suggested by the presence of binding sites for the immediate early genes Myc/Max (-941, -

291), Activator Protein (AP)-1 (-3116, -1678, -454, -271, -204), and N-Myc (-292).

A comparison of the 5' UTR and proximal promoter of mouse *SCD1* and human *SCD* reveal sequence identity of 64 % between –298 to +1, suggestive of conserved regulatory elements (Fig. 2). The presence and position of the following elements is common to mouse *SCD1* and human *SCD* promoters: TATA boxes, binding sites for Aryl Hydrocarbon Receptor (ARNT), Myc/Max, Whn, AP-4, GATA-1, Gut-Enriched Krueppel-like Factor (GKLF), and AP-1. Also common is the FSE2, which contains binding sites for VDR, Ets-1, HNF3-β, as well as a binding site for C/EBP-αan element in the mouse SCD1 promoter that binds C/EBP protein upon differentiation of 3T3-L1 cells (Christy *et al.*, 1989) Furthermore, the NF-1 site (-245) that is required for mouse *SCD1* transcription (Singh and Ntambi, 1998), is present in the human *SCD* promoter. The notable differences between the promoters in this region are the presence in the human of Rel, NF-kB, N-Myc, and RFX-1 binding sites.

EXAMPLE 10: Identification of the Transcription Initiation Site

Screening of a cDNA library from human foreskin keratinocytes for *SCD* resulted in 2 clones which extend 5' from the ATG translation initiation, these being clone 34 to –235 and clone 45 to –229(Zhang *et al.*, 1999) (arrowheads, Fig. 1). To further define the start site for transcription in skin cells, primer extension and Rnase protection were carried out with total RNA isolated from hair follicle biopsies and cultured HaCaT keratinocytes. Due to the high GC content in this region, primer extension did not yield consistent results. For the RNase protection assays, a cRNA probe, consisting of 302 nt of *SCD* (excluding vector sequence) from -275 to +27 was used in the protection assay. A major band of 172 nt was detected in total RNA isolated from hair follicles, whereas a major band of 300 nt was seen in HaCaT keratinocyte total RNA (Fig. 3). No bands of the size of full length probe (400 nt, including vector sequence) were detected (indicating lack of contaminating sense DNA template and in vitro transcribed sense RNA) nor was any

smearing evident (indicating complete Rnase digestion). Liver showed the same protected fragments as hair follicles, whereas brain had a similar protection pattern to that of HaCaT cells (data not shown). This indicates that for hair follicles and liver, the major site of transcription initiation is at –145 (solid box in Fig. 1) from the translation start site. This position is 35 nt downstream of the proximal TATA box at –179 nt, similar to mouse *SCD*1 in 3T3-L1 cells (Fig. 2; ref (Ntambi *et al.*, 1988)). In cultured keratinocytes, the transcription start site is 5' to that of hair follicles, as indicated by the 300 nt protected fragment in HaCaT cell RNA and the termination of cDNA clones 34 and 45 in the region of the distal TATA box located at –234 nt (Fig. 3). There are several minor protected bands in HaCaT cells and skin, with a common band of 220 nt (Fig. 3). This indicates the presence of a minor transcription start site at –193, a position 42 nt downstream of the distal TATA box (single-lined, dashed box in Fig. 1). Brain RNA shows an identical protection pattern that of HaCaT cells (data not shown). Overall, these results suggest that the *SCD* gene has different major transcription start sites that depend on tissue specific factors.

EXAMPLE 11: Functional Analysis of the *SCD* promoter

To determine if the sequences upstream of the transcription initiation site are sufficient to direct transcription, a 3.2 kb *Pst*I-*Sac*II fragment of the 5' flanking region was fused in both orientations to a promoterless luciferase reporter gene and examined for the ability to mediate basal transcription. In addition, progressive 3' deletions in the putative promoter region were also examined for their effect on reporter activity. The resulting constructs were transiently transfected into the human keratinocyte cell line, HaCaT, and cell extracts were assayed for luciferase activity at 48h post-transfection. A total 7 different constructs were initially generated using convenient restriction enzyme sites in the 3.2 kb flanking region (Fig. 4). Significant transcriptional activity was contained with 5 constructs covering the region between –3348 to –882 (Fig. 4). Further deletion to -461 resulted in complete loss of activity (Fig. 4). *SCD* sequences fused in opposite transcriptional orientation did not induce any

significant level of reporter activity (Fig. 4). The highest promoter activity was obtained with construct c5 which corresponded to bp –882 to –150 (Fig. 4). These results demonstrated that, of the constructs tested, c5 contained the minimal required nucleotide sequence information for *SCD* transcription, and that essential cis elements lay between –882 and –461.

To further delineate cis elements responsible for human *SCD* gene expression, the c5 construct was deleted further to create three more constructs, c8, c9, and c10 (Fig. 5). These construct were transiently transfected into HaCaT cells, in addition to the parental construct c5. Deletion of 130 nt from the 5'-end of the c5 construct (to generate c8) did not have any major effect on the promoter activity. Similarly, deletion of a further 144 nt, to generate c9, did not affect *SCD* promoter activity. However, deletion of a further 113 nt, to generate c10, completely removed the putative core promoter region as indicated by the reduced luciferase activity to background level (Fig. 5). Taken together, these results narrowed the region that contains essential cis elements required for *SCD* transcription in HaCaT keratinocytes to –609 to -498.

Examination of the sequences of this region revealed several potential transcription factor binding sites (Fig. 5, lower panel); AP-2 (-594), ISRE (-561), SP-1 (-551), RFX-1 (-522, -495), SREBP (-517), NF-1 (-502), and NF-Y (-501). Considering the role SP-1 and NF-Y in regulation of the Fatty Acid Synthase promoter (Roder *et al.*, 1997), and NF-1 (Singh and Ntambi, 1998) and NF-Y (Tabor *et al.*, 1999) in the regulation of mouse *SCD* genes, the effect of mutations in the SP-1 and CCAAT box on *SCD* promoter activity was determined using site-directed mutagenesis. Using c9, the core binding site for SP-1 at –551 was changed from GGAG to AAAG (boxed in Fig. 5) and the CCAAT box binding site for NF-1/NF-Y at –502/-501 was changed from CCAA to AAAA (boxed in Fig. 5). Relative to the promoter activity of c9, mutation of the SP-1 site has no effect (Fig. 6). However, mutation of the CCAAT box completely removes *SCD* promoter activity (Fig. 6). These results indicate that a critical cis element for *SCD* promoter activity is the CCAAT box located at –501.

EXAMPLE 12: EMSA for CCAAT Box Binding Proteins

To determine if the CCAAT box critical for *SCD* promoter activity was actually occupied by transcription factors present in HaCaT cell nuclei, we prepared nuclear extracts and performed EMSA (Fig. 7). A specific gel shift was obtained when nuclear extract from HaCaT cells was assayed with radiolabeled oligonucleotide corresponding to the CCAAT box binding site at –501 nt. The gel shifts were competed with non-radiolabeled CCAAT oligonucleotide, but not the AP-2 (-594) and SP-1 (-551) oligonucleotides. There are a few lower bands that were partially competed by CCAAT oligonucleotide but not by the AP-2 and Sp-1 oligonucleotides. Since the CCAAT oligonucleotide contains binding sites for 2 CCAAT box binding proteins, NF-1 and NF-Y, as well as a binding site for the X box binding protein, RFX-1, this suggests the possibility that the specific bands of different mobility are due to different complexes of these three transcription factors.

EXAMPLE 13: Regulation of the *SCD* Promoter by Unsaturated Fatty Acids

Previous work identified a region of the mouse *SCD1 and SCD2* promoter that was responsible for down-regulation of promoter activity by UFA's (Waters *et al.*, 1997b). This region of the human *SCD* promoter was compared to that of mouse *SCD1* and *SCD2* (Fig. 8). A striking degree of sequence similarity (99%) and identity (77%) between the three *SCD* genes is present in this region, suggesting conservation of important regulatory elements. It is notable that critical cis elements for mouse *SCD1/SCD2* promoter activity, namely the CCAAT box element at -502/-501 (NF-1/NF-Y binding site) and the novel SRE at –517 are located within this region, and are conserved among all three genes. In addition, several other transcription factor binding sites are conserved among all three genes, these being IK2, AP-4, and RFX-1. To examine if the minimal *SCD* promoter (containing the UFA response region) could respond to UFA, c9 was transfected into HaCaT keratinocytes, and the cells subsequently exposed to various fatty acids conjugated to BSA (Fig. 9). BSA alone had no effect on *SCD* promoter activity. In contrast, stearate

unregulated promoter activity, whereas oleate (a MUFA) and linoleate (a PUFA) down regulated promoter activity to the same extent. It is notable that stearate and oleate are the substrate and product, respectively, of the *SCD* enzyme. These results indicate that PUFA down-regulates transcription of human *SCD*, similar to mouse *SCD1* and *SCD2*. Moreover, it shows that the substrate and product of the *SCD* enzyme can regulate transcription of the *SCD* promoter, through activation and repression, respectively.

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It is understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the claims.

What is claimed is: